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Decreased Numbers of Circulating B Cells in Myeloma Patients With Reduction After Conventional Chemotherapy

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Reports of high numbers of circulating monotypic B cells in patients with multiple myeloma (MM) have recently been published. These cells, which were identified by their expression of CD19, were reported to be resistant to conventional chemotherapy and to represent the source of relapse. We examined blood samples from 48 patients before and 53 patients after glucocorticoid containing chemotherapy by dual color flow cytometry. The absolute count of $CD19^+$ B cells in patients before treatment ($212.6 \pm 24.8 \times 10^6$/l) was decreased compared to normal controls ($P = .038$). In the post-treatment group, circulating B cells were highly significantly lower than in untreated patients ($45.23 \pm 6.69 \times 10^6$/l. $P < .001$). This reduction was also seen in 26 patients, that were followed during chemotherapy. The cytoplasmic κ/λ ratio was within normal range before and after treatment with no difference according to the light chain isotype of the paraprotein. We conclude that circulating B cells are not increased in patients with MM, that the majority of these cells are polyclonal, and that conventional chemotherapy effectively reduces circulating B cells without leading to dominance of resistant monotypic cells. Am. J. Hematol. 57:287–292, 1998.

Key words: multiple myeloma; B cells; chemotherapy

INTRODUCTION

Multiple myeloma (MM) has been considered a neoplasia of plasma cells, characterized by the infiltration of bone marrow with a clone of plasma cells and the production of a monoclonal immunoglobulin (Ig). Over recent years, evidence has accumulated of the neoplastic involvement of earlier cells of B lineage in this disease. It is believed now that the oncogenic event occurs at pre-B or B cell-level and that the cells follow the normal differentiation pathway to plasma cells [1–3], expressing increasing densities of CD38 while losing CD19, CD45 and surface expression of Ig [4–6]. In the peripheral blood (PB) of MM patients, B cells from the tumor lineage have been discovered by different methods, most recently by Ig-gene-analysis [7–10].

There is still much controversy about the extent of B cell involvement. Some authors reported highly elevated numbers of B cells in the PB of MM patients [11,12]. These cells were considered to be monoclonal with surface and cytoplasmic light chain restriction according to the paraprotein isotype and an abnormal phenotype with expression of CD45R0, CD56, and a high density of CD38 [13]. Other investigators have found low to normal numbers of B cells [7,14,15] with normal light chain ratio [16,17] or an excess of the uninvolved light chain, which may represent a suppressor mechanism in patients with stable disease [18–20]. Furthermore, circulating monoclonal B cells have been suggested to be the source of relapse in MM. According to Pilarski et al. [21], these cells are not reduced by chemotherapy due to their expression of the multidrug resistance complex.

As we treat eligible patients with advanced MM with high-dose chemotherapy followed by blood stem cell transplantation, the possible existence of a large resistant tumor population in the peripheral blood of MM patients is of great interest to us. We, therefore, determined the number of $CD19^+$ B cells in the PB of MM patients

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TABLE I. Patient Characteristics

	Patients prior to chemotherapy (n = 48)	Patients post-chemotherapy (n = 53)
Isotype	IgG 53%	IgG 60%
	Bence Jones 24%	Bence Jones 22%
	IgA 19%	IgA 16%
	IgD 4%	IgD 2%
Light chain	κ 62%	κ58%
	λ 38%	λ 42%
Stage (Salmon & Durie)	I 6%	I 9%
	II 24%	II 18%
	III 70%	III 73%
Male/female	1.29	1.25
Age (mean ± SE)	52.19 ± 1.21	52.62 ± 1.42

before and after conventional chemotherapy by dual color flow cytometry.

MATERIALS AND METHODS

Patients

After informed consent blood samples were drawn from 101 MM patients and 21 healthy volunteers. Forty-eight untreated patients were investigated prior to glucocorticoid containing chemotherapy. Twenty-six of these patients were analyzed after chemotherapy as well. Fifty-three patients were investigated 4 weeks after their last cycle of chemotherapy. Of these 38 had received VAD (vincristine, adriamycine®, and dexamethasone), 10 MP (melphalan and prednisone), and 5 M2 (vincristine, carmustine, melphalan, cyclophosphamide, and prednisone). The mean number of cycles received was 6.16 ± 0.61. (For patient characteristics see Table I.)

Preparation

Mononuclear cells were prepared by Ficoll-Hypaque density centrifugation and resuspended in 1% PBS to a concentration of 1.5–2 × 10^{10}/l. Subsequently, 50-μl of the cell suspension was incubated for 20 min at 4°C with combinations of the following antibodies: mouse IgG1 FITC and PE isotype control, CD19 FITC and PE, CD38 FITC and PE, CD45 FITC, CD11b PE, CD14 PE (Immunotech, Marseille, France), CD13 PE, anti-human κ and λ light chain FITC (Dianova, Hamburg, Germany), CD19 FITC (clone B4, Coulter, Miami, FL). For the lysis of residual erythrocytes, OptiLyse B solution (Immunotech) was used according to the manufacturer's instructions. For intracellular light chain staining, FIX&PERM (An der Grub, Kaumberg, Austria) was used as recommended by the manufacturer.

Analysis

Cells (50,000) were measured with a FACScan Cytometer (Becton Dickinson, Heidelberg, Germany) using a live gate to exclude debris and neutrophils. Two gates were set in the forward-scatter (FSC) side-scatter (SSC) dot plot of Cellquest II Software (Becton Dickinson): a lymphocyte gate based on $CD45^+/CD14^-$ expression and a plasma cell gate containing all cells with low SSC [4].

In order to obtain absolute cell counts, percentages of flow cytometric analysis were multiplied by the absolute numbers of lymphocytes derived from a differential white cell count from the same day for lymphocyte gated cells and by absolute numbers of leukocytes without neutrophils (lost through Ficoll-Hypaque and live gating) for cells of the plasma cell gate. The κ/λ ratio was computed as fraction of CD19 coexpressing cells in the lymphocyte gate.

TABLE II. Relative and Absolute Numbers of $CD19^+$ B Cells in the Lymphocyte Gate, and $CD38^{++}$ Cells and $CD38^{++}/CD19^+$ Coexpressing Cells in the Plasma Cell Gate

	Absolute CD19 (×10^6/l)	Absolute $CD38^{++}$ (×10^6/l)	Absolute $CD38^{++}/CD19^+$ (×10^6/l)
Prior to therapy	212.6 ± 24.8	11.2 ± 3.3	1.00 ± 0.27
Post-therapy	45.23 ± 6.69	11.9 ± 5.5	0.82 ± 0.21
Normal controls	281.4 ± 33.2	1.93 ± 0.52	1.00 ± 0.44
P (prior vs. normal)	.038	.004	.842
P (prior vs. post)	<.001	.137	.863

Statistical Analysis

Results are expressed as mean ± standard error of the absolute number of cells per liter. To test for significant differences between the groups of patients and normal persons, the Mann-Whitney U-test was used. In the patients that were followed, values were compared by the Wilcoxon matched pairs test. All tests were done using the software Statistica®.

RESULTS

CD19 (B4)

We tested the monoclonal antibody CD19 FITC (clone B4, Coulter) in 14 normal controls and 23 MM patients. We found significantly higher values (33.4 ± 2.2%) in both groups compared to CD19 (Immunotech) ($P < .001$) with the plasma cell gate, which always includes myelomonocytic cells. $CD19\ (B4)^+$ cells showed a high coexpression of myelomonocytic antigens ($CD11b^+$ 20.9 ± 2.1%; $CD13^+$ 20.6 ± 1.9%; $CD14^+$ 16.4 ± 1.8%) in MM patients as well as in normal controls (Fig. 1). The percentage of $CD19\ (B4)^+$ cells that coexpressed CD11b was not different in MM patients compared to normal persons ($P = .162$). Also, the percentage of cells coex-

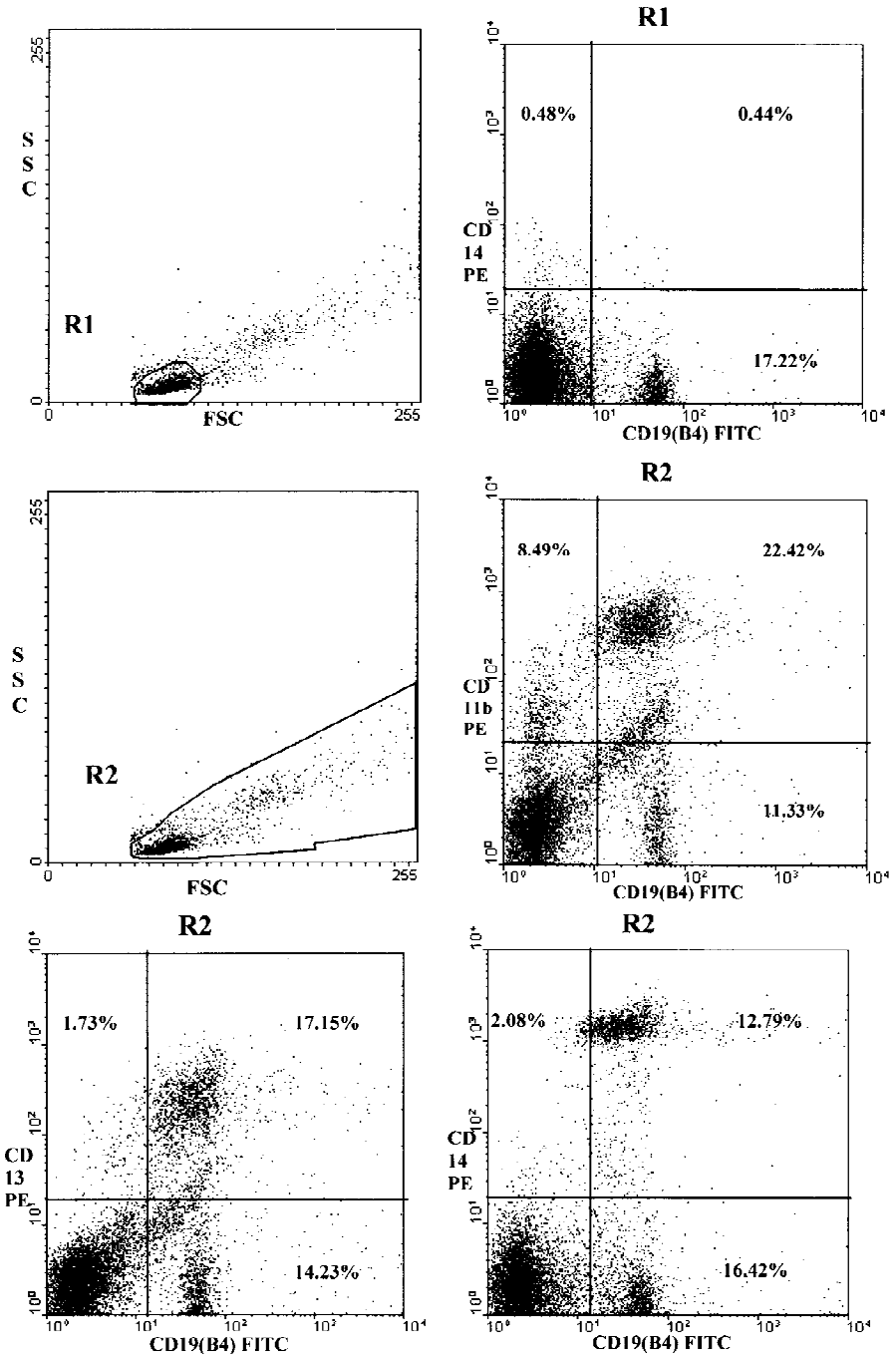

Fig. 1. Evaluation of the antibody CD19 (B4, Coulter) in a normal control. Top: Normal percentage of CD19$^+$ cells in the lymphocyte gate. Middle and Bottom: In the plasma cell gate, CD19 (B4) stains large numbers of cells bearing myelomonocytic antigens (CD11b, CD13, CD14).

pressing high densities of CD38 showed no significant difference ($P = .294$). The percentage of CD19 (Immunotech)$^+$ cells (11.5 ± 1.3%) was not significantly different from CD19 (B4)$^+$/CD11b$^-$ (12.6 ± 1.6%, $P = .289$) or CD19 (B4)$^+$/CD13$^-$ cells (12.8 ± 1.4%, $P = .167$). The results reported below were obtained using CD19 (Immunotech) to avoid staining of myelomonocytic cells.

CD19$^+$ B Cells

For untreated patients, the absolute number of CD19$^+$ B cells was 212.6 ± 24; 8 × 10^6/l compared to 281.4 ± 33.28 × 10^6/l for normal controls ($P = .038$). Patients 4 weeks after chemotherapy had a highly significantly lower number of B cells (45.23 ± 6.69 × 10^6/l, $P < .001$).

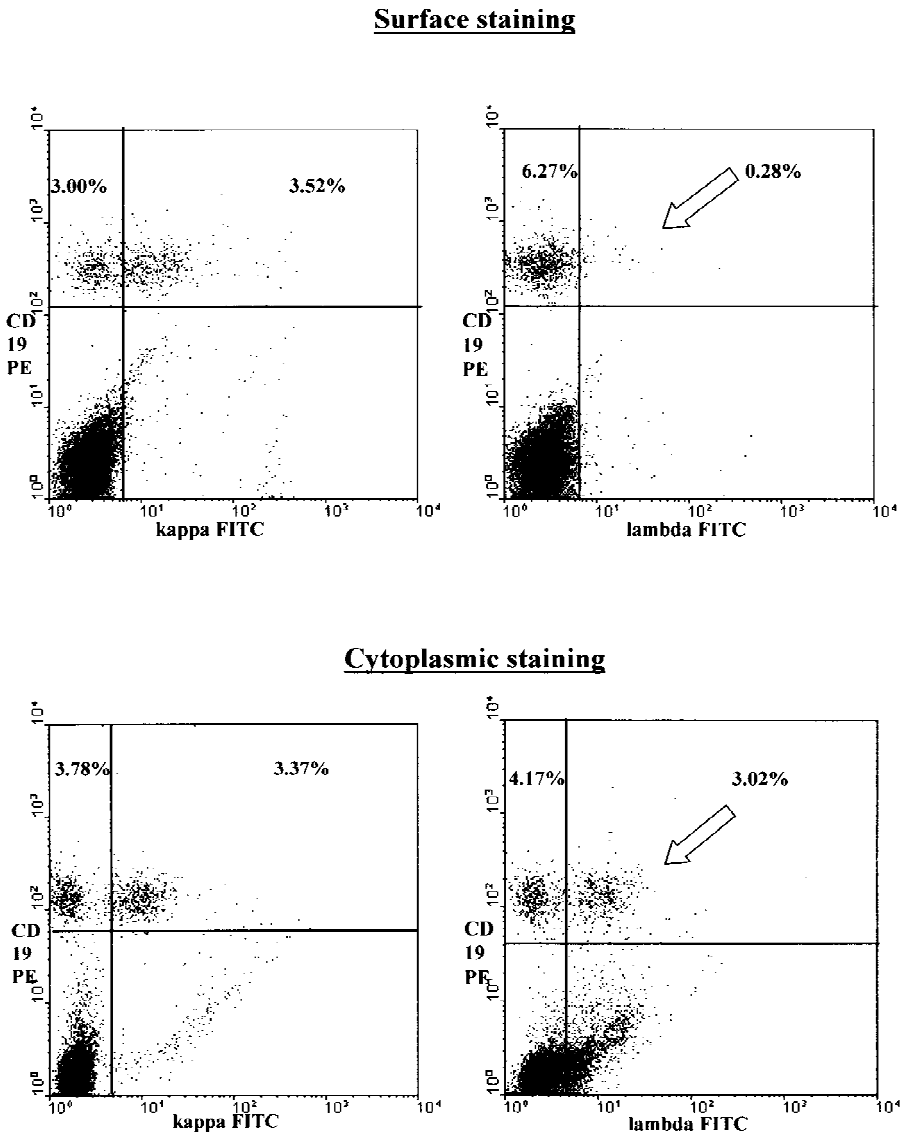

Fig. 2. Surface and cytoplasmic staining with antibodies against light chains and CD19 in a MM patient with paraprotein isotype IgG-λ before chemotherapy. Top: Surface staining; (κ/λ = 10.5). Bottom: Cytoplasmic staining: (normal κ/λ ratio of 1.16).

The reduction of B cells by chemotherapy was confirmed in 26 patients that were analyzed sequentially at diagnosis (222.4 ± 36.9 × 10^6/l) and after 3 cycles of chemotherapy (106.3 ± 27.4 × 10^6/l. $P = .025$).

CD38^{++} Cells

The absolute count of polyclonal CD38^{++} cells in patients prior to chemotherapy was significantly higher than in normal controls (11.17 ± 3.28 × 10^6/l vs. 1.93 ± 0.52 × 10^6/l, $P = .004$). The number seen in the post-treatment group was not significantly different compared to untreated patients (11.99 ± 5.56 × 10^6/l, $P = .137$). In the follow up-group we did not find a significant reduction of circulating CD38^{++} cells after chemotherapy ($P = .743$).

Prior to therapy, MM patients had 1.00 ± 0.27 × 10^6 cells per liter that coexpressed CD38^{++}/CD19^{+}. This was not significantly different from normal controls ($P = .842$). There was no significant change seen after chemotherapy ($P = .863$) (See Table II).

κ/λ Ratio

In 21 normal controls we found a surface κ/λ ratio ranging from 0.79 to 2.17 (1.26 ± 0.06) and a cytoplasmic κ/λ ratio ranging from 1.15 to 1.82 (1.44 ± 0.04). In MM patients the κ/λ ratio differed significantly between surface and cytoplasmic staining (Fig. 2). With surface

staining, we found a κ/λ ratio of 0.64 ± 0.12 for κ-type myeloma and a ratio of 3.96 ± 1.45 for λ-type myeloma. We could not detect a correlation between the extent of surface light chain isotype suppression and amount of beta2-microglobulin or stage of disease. By cytoplasmic staining we found a ratio of 1.10 ± 0.22 for κ-type myeloma and a ratio of 1.25 ± 0.10 for λ-type myeloma, with no significant change after chemotherapy ($P\kappa = .197$; $P\lambda = .837$). The intracellular κ/λ ratio did not differ significantly according to the light chain type of paraprotein.

DISCUSSION

It has recently been reported that myeloma patients have 2 to 8 times as many circulating $CD19^+$ cells as normal controls, which are monotypic as defined by cytoplasmic or surface light chain restriction and show an abnormal phenotype with coexpression of CD45R0, CD56, and high densities of CD38 [13]. It has also been reported that these cells are not reduced by chemotherapy and form a drug-resistant reservoir responsible for relapse in MM [21]. The authors of these reports stressed the importance of using CD19 FITC (B4, Coulter), because the abnormal circulating B cells in MM were not reliably detected with other commercially available antibodies or with PE conjugates because of steric hindrance. Testing CD19(B4)FITC we also found highly elevated numbers of positive cells. However, about 60% of these cells coexpressed myelomonocytic antigens (CD11b, CD13, CD14). This was seen in myeloma patients as well as in normal persons, indicating that the antibody does not detect a subtype of B cells typical of myeloma, but also myelomonocytic cells. In our study, coexpression of $CD38^{++}$ on these cells did not differ between myeloma patients and normal controls. As it is necessary to use a large gate, which always includes monocytes, for the detection of plasma cells, we prefer CD19 (Immunotech), which shows no staining of myelomonocytic cells. Leu-12 (Becton Dickinson) or B4 (Coulter) as a PE-conjugate also do not show staining of monocytes and the results are comparable to CD19 (Immunotech) (data not shown).

Using these antibodies, our results show significantly lower numbers of $CD19^+$ B cells in PB of untreated MM compared to normal controls. This is in keeping with results published by other authors, who used Leu-12 (Becton Dickinson) [14], FMC63 (not commercially available) [7], or B4 (Coulter) with restriction to the lymphocyte gate [15].

In patients after glucocorticoid containing chemotherapy, we found the number of B cells decreased even more. In a follow-up study of patients, we noted a reduction of $CD19^+$ cells in the blood by about 52% after chemotherapy compared to pre-treatment values. This agrees with results published by Lemoli et al. [22], who observed 2.4 ± 2.2% of B cells in patients prior to mobilization therapy for blood stem cell transplantation using Leu-12 antibody (Becton Dickinson), and those reported by Kay et al. [23], who found B cell levels reduced by 51% in patients treated with VBMCP and variations.

In contrast with the values reported by Bergsagel et al. [13], our studies showed very low numbers of circulating premature plasma cells, characterized by coexpression of $CD38^{++}/CD19^+$, in MM patients. On an average, they represented 20 cells in 50,000 analyzed, with only one patient showing more than a hundred. We could not detect a difference between normal controls, pre-treatment or post-treatment values.

When analyzing the κ/λ ratio, we found a large difference between the surface and intracellular staining of $CD19^+$ cells. The cytoplasmic ratio was not different from normal controls for patients before and after treatment. This indicates that the majority of B cells are polyclonal. This is supported by Chen and Epstein and Billadeau et al. [7,10]; and by studies of our laboratory using patient-specific CDR3-PCR, which showed that less than 1% of $CD19^+$ cells were clonally related to the tumor clone [24].

The fact that the κ/λ ratio was unchanged after treatment supports the hypothesis that chemotherapy does not selectively spare monotypic B cells, since this would result in the relative dominance of B cells with the paraprotein light chain isotype. Our observation makes the existence of a large clone of B cells that is resistant to chemotherapy unlikely.

On the cell surface we observed a significant loss of light chain expression of the paraprotein isotype, resulting in a low κ/λ ratio for κ-myeloma and a high ratio for λ-myeloma. This phenomenon has also been observed by others [18–20], though only in patients with stable disease, whereas our patients were all in need of treatment. However, our results indicate that this is not due to a loss of B cells producing the paraprotein light chain, but only to a loss of surface expression on these cells, which retain the ability to produce intracellular polyclonal immunoglobulins.

We conclude that the number of circulating B cells in patients with advanced MM is lower than in normal controls. After treatment with glucocorticoid containing chemotherapy, B cells are significantly reduced. Using intracellular light chain staining, the cells appear to be polyclonal as they neither showed a clonal excess nor a suppression of the paraprotein light chain.

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